

The University of Chicago

# STUDIES ON BIOLOGICAL CONDITIONING OF WATER

- I. MODIFIED VAN SLYKE PROCEDURE FOR OXYGEN AND CARBON DIOXIDE DETERMINATIONS ON WATER
- II. THE RÔLE OF CALCIUM IN BIOLOGICAL CONDITIONING
- III. MODIFIED PROCEDURE FOR DETERMINING THE NITROGEN DISTRIBUTION OF PROTEINS INVOLVED

A DISSERTATION SUBMITTED TO THE FACULTY OF THE  
DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY  
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY  
AND PHARMACOLOGY

1935

By

RALPH B. OESTING

Private Edition, Distributed by  
THE UNIVERSITY OF CHICAGO LIBRARIES  
CHICAGO, ILLINOIS

Reprinted from

PHYSIOLOGICAL ZOÖLOGY, Vol. VII, No. 4, October 1934  
BIOLOGICAL BULLETIN, Vol. LXVIII, No. 2, April 1935



The University of Chicago

# STUDIES ON BIOLOGICAL CONDITIONING OF WATER

- I. MODIFIED VAN SLYKE PROCEDURE FOR OXYGEN AND CARBON DIOXIDE DETERMINATIONS ON WATER
- II. THE RÔLE OF CALCIUM IN BIOLOGICAL CONDITIONING
- III. MODIFIED PROCEDURE FOR DETERMINING THE NITROGEN DISTRIBUTION OF PROTEINS INVOLVED

A DISSERTATION SUBMITTED TO THE FACULTY OF THE  
DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY  
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY  
AND PHARMACOLOGY

1935

By  
RALPH B. OESTING

Private Edition, Distributed by  
THE UNIVERSITY OF CHICAGO LIBRARIES  
CHICAGO, ILLINOIS

---

Reprinted from  
PHYSIOLOGICAL ZOOLOGY, Vol. VII, No. 4, October 1934  
BIOLOGICAL BULLETIN, Vol. LXVIII, No. 2, April 1935





Reprinted from  
PHYSIOLOGICAL ZOOLOGY, Vol. VII, No. 4, October, 1934  
PRINTED IN THE U.S.A.

---

R. B. OESTING

# A MODIFIED VAN SLYKE METHOD FOR THE DETERMINATION OF DISSOLVED OXYGEN AND TOTAL CARBON DIOXIDE IN WATER<sup>1</sup>

(One figure)

R. B. OESTING

Whitman Laboratory of Experimental Zoölogy, University of Chicago

THE need in aquatic respiration studies for determinations of dissolved oxygen and total carbon dioxide in small samples of water led the author to modify the Van Slyke and Neill (1924) manometric procedure for the analysis of the oxygen and carbon dioxide content of the blood to serve this purpose. Van Slyke and Neill (1924) have also pointed out a procedure adapting this apparatus to the analysis of water. The difficulties encountered in the Winkler method from nitrites and dissolved organic matter are well known. The Van Slyke procedure is free from these and has the advantage of requiring only a 5 cc. sample for an oxygen and carbon dioxide determination.

## APPARATUS<sup>2</sup>

The apparatus used was of the portable closed manometer type described by Van Slyke (1927). The samples were measured into the reaction chamber with 5 cc. Ostwald-Van Slyke pipettes. A very satisfactory gas-free reagent container, designed and used in the laboratory of Dr. M. E. Hanke, was employed instead of those described by Van Slyke and Neill. Figure 1 will illustrate.

The tip of this apparatus is dipped into a small test tube filled with enough mercury to form a seal.

## REAGENTS

*Five per cent lactic acid.*—Dilute 5 cc. of the C.P. (85 per cent) lactic acid to 100 cc. with distilled water.

<sup>1</sup> This work was supported by a grant administered by Dr. W. C. Allee at the University of Chicago from the Rockefeller Foundation to aid researches in the biological sciences. The author wishes to express his sincere appreciation to Drs. W. C. Allee and M. E. Hanke for their suggestions.

<sup>2</sup> Manufactured by Eimer and Amend, New York.

*Gas-free 2N NaOH reagent.*—Extract the dissolved gases from the reagent in the Van Slyke apparatus and store in a gas-free reagent container (see Van Slyke and Neill, 1924).

*Gas-free hydrosulphite reagent.*—Dissolve 10 gm. of sodium hydrosulphite plus 1 gm. of sodium anthraquinone-sulfonate in 50 cc. of 1N KOH in a bottle just large enough, when stoppered, to contain the reagent. Transfer by decantation to the reaction chamber of the Van Slyke apparatus. Extract the dissolved gases and store in a gas-free reagent container. At 30° this reagent will keep for a week; at lower temperatures it will keep for 2 weeks or more, as Sendroy states (1931).

#### PROCEDURES

In describing the procedures, the stopcock at the top of the reaction chamber will be designated as stopcock *b* and the one controlling the mercury level in the apparatus as stopcock *e*. Van Slyke's articles describe the apparatus and the precautions to be used in its manipulation. This procedure is essentially that described by Van Slyke and Neill (1924). The only modifications are concerned with the concentrations of reagents used which have been altered to suit the special conditions demanded by the analysis of water, but so chosen that the final concentrations are approximately the same as those used by Van Slyke and Neill.

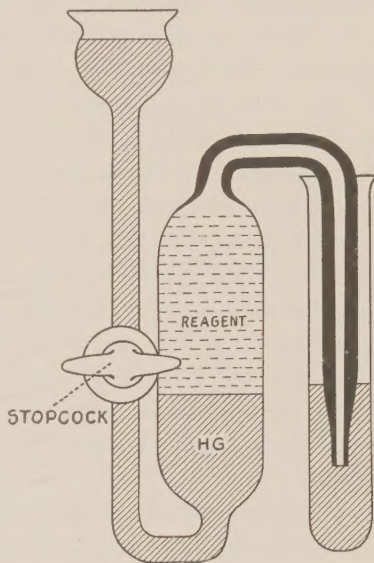


FIG. 1.—Gas-free reagent container

#### A. FOR THE BLANK

1. Have the reaction chamber filled with mercury, stopcock *b* closed, *e* open, and the leveling bulb at the lower level.
2. By means of graduations on the cup, introduce 6 cc. of distilled water, followed by 1 cc. of 5 per cent lactic acid, into the reaction chamber. Seal stopcock *b* with mercury.



3. Lower the mercury level to the 50 cc. mark by means of the leveling bulb and stopcock *e*, and shake for 3 minutes. Raise the liquid and expel the gas extracted and 0.5 cc. of the acid water mixture. Again seal stopcock *b* with mercury and lower to the 50 cc. mark and shake for 2 minutes.

4. Raise the liquid and expel the gas extracted and 1 cc. of the acid water mixture, leaving 5.5 cc. in the chamber. Reseal stopcock *b* with mercury, lower to the 50 cc. mark, and shake for 2 minutes.

5. Raise the meniscus level of the acid water mixture rapidly at first, and then slowly and evenly to the 0.5 cc. mark, and read  $p_1$  to 0.1 mm. on the manometer. Lower to the 50 cc. mark, shake 1 minute, and repeat the  $p_1$  reading. These should check to 0.5 mm., or the procedure should be repeated until checks are obtained.

6. Lower the liquid level to just below the 2 cc. mark; introduce 1 cc. of the gas-free NaOH into the cup and 0.5 cc. slowly into the chamber. Seal stopcock *b* with mercury and, after 30 seconds for absorption and drainage, raise the meniscus slowly to the 0.5 cc. mark and take the  $p_2$  reading. Repeat this reading by lowering the meniscus below the 2 cc. mark and again setting it at the 0.5 cc. mark. These readings should check to 0.1 mm.

7. After the  $p_2$  reading, record the temperature of the water jacket.

8. Introduce 1 cc. of the hydrosulphite reagent into the cup and 0.5 cc. slowly into the chamber. Seal stopcock *b* with mercury and raise the liquid level to the top of the chamber. Now lower the meniscus below the 2 cc. mark and allow 30 seconds for absorption and drainage. Raise the meniscus slowly to the 0.5 cc. mark and take the  $p_3$  reading. Repeat this reading, as was done with  $p_2$ .

9. Wash out the chamber with 10 per cent lactic acid. Rinse with distilled water and it is ready to use again.

#### B. FOR ANALYSIS OF CO<sub>2</sub> AND O<sub>2</sub> TOGETHER

1. Introduce 2.5 cc. of 5 per cent lactic acid into the cup and 2 cc. into the chamber. Seal stopcock *b* with mercury and lower the mercury level in the chamber to the 50 cc. mark. Shake for 3 minutes. Raise and expel the gas extracted and the liquid into the cup, leaving the capillary filled with mercury.

2. Introduce the 5 cc. sample into the chamber as follows: Allow



the evacuated space at top of the manometer to become completely filled with mercury, and close stopcock *e*. Then place the 5 cc. pipette containing the sample into the cup, obtaining a seal at the bottom by means of a rubber tip fitted to the end of the pipette. Open first the

TABLE I

FACTORS FOR CONVERTING THE PRESSURE READINGS TO  
CC. OF GAS PER LITER OF WATER, FOR WATER CON-  
TAINING SMALL AMOUNTS OF CARBONATES

Size of sample, 5 cc.

Total volume of solution, 5.5 cc.

Volume of gas, 0.5 cc.

| Temp. (Degrees C.) | CO <sub>2</sub> | O <sub>2</sub> |
|--------------------|-----------------|----------------|
| 15.....            | 0.1443          | 0.1248         |
| 16.....            | 0.1433          | 0.1244         |
| 17.....            | 0.1424          | 0.1239         |
| 18.....            | 0.1415          | 0.1235         |
| 19.....            | 0.1407          | 0.1230         |
| 20.....            | 0.1399          | 0.1226         |
| 21.....            | 0.1391          | 0.1222         |
| 22.....            | 0.1382          | 0.1217         |
| 23.....            | 0.1374          | 0.1213         |
| 24.....            | 0.1366          | 0.1208         |
| 25.....            | 0.1358          | 0.1204         |
| 26.....            | 0.1351          | 0.1200         |
| 27.....            | 0.1344          | 0.1195         |
| 28.....            | 0.1337          | 0.1191         |
| 29.....            | 0.1330          | 0.1187         |
| 30.....            | 0.1323          | 0.1183         |
| 31.....            | 0.1316          | 0.1179         |
| 32.....            | 0.1310          | 0.1175         |
| 33.....            | 0.1304          | 0.1171         |
| 34.....            | 0.1297          | 0.1168         |

pipette stopcock and then stopcock *b*, holding the pipette firmly in place. Next, by means of stopcock *e*, control the flow of the sample into the chamber and, when the meniscus is near the low mark of the pipette, close stopcock *e*. The final adjustment of the liquid in the pipette is made by exerting a variable pressure with the pipette, and closing the stopcock of the pipette when the meniscus is exactly at the lower mark. Stopcock *b* is then closed. Now open stopcock *e* and gently raise the pipette 1 or 2 mm. above the bottom of the cup, and,

holding it in this position, allow 0.5 cc. of the 5 per cent lactic acid to flow into the chamber through stopcock *b*, thus washing in the last of the sample around the tip of the pipette. Remove the pipette and seal stopcock *b* with mercury. Lower the mercury level to the 50 c.c. mark and shake for 3 minutes.

3. Proceed as directed under steps 5-9, inclusive, of the blank (A)

TABLE II

FACTORS FOR CONVERTING THE PRESSURE READINGS TO CC. OF  
GAS PER LITER OF WATER FOR WATERS CONTAINING  
LARGE AMOUNTS OF CARBONATES

Size of sample, 5 cc.

Total volume of solution, 5.5 cc.

Volume of gas, 2.0 cc.

| Temp. (Degrees C.) | CO <sub>2</sub> | Temp. (Degrees C.) | CO <sub>2</sub> |
|--------------------|-----------------|--------------------|-----------------|
| 15.....            | 0.5661          | 25.....            | 0.5329          |
| 16.....            | 0.5624          | 26.....            | 0.5300          |
| 17.....            | 0.5588          | 27.....            | 0.5271          |
| 18.....            | 0.5552          | 28.....            | 0.5244          |
| 19.....            | 0.5530          | 29.....            | 0.5217          |
| 20.....            | 0.5487          | 30.....            | 0.5190          |
| 21.....            | 0.5456          | 31.....            | 0.5164          |
| 22.....            | 0.5423          | 32.....            | 0.5140          |
| 23.....            | 0.5391          | 33.....            | 0.5114          |
| 24.....            | 0.5360          | 34.....            | 0.5090          |

#### C. FOR ANALYSIS OF CO<sub>2</sub> ALONE

Proceed as directed under steps 1 and 2 of B. Then follow steps 5, 6, 7, and 9 of the blank (A). Omit step 8.

#### D. FOR ANALYSIS OF O<sub>2</sub> ALONE

Proceed as directed under steps 1 and 2 of B, shaking for 4 minutes instead of 3 at the end of step 2. Raise to just below the 2 cc. mark and continue as directed under steps 6, 7, 8, and 9 of the blank (A). Omit step 5.

#### CALCULATIONS

$$p_1 - p_2 (\text{for A}) = C_{CO_2}, \quad (p_1 - p_2 - C_{CO_2}) \times \text{Factor}_{CO_2} = \text{cc. of CO}_2 \text{ per L.}$$

$$p_2 - p_3 (\text{for A}) = C_{O_2}, \quad (p_2 - p_3 - C_{O_2}) \times \text{Factor}_{O_2} = \text{cc. of O}_2 \text{ per L.}$$

Factor  $\text{CO}_2$  and Factor  $\text{O}_2$  are taken from Table I. This table was calculated from the equations and data given in the papers from Van Slyke and his co-workers (1924 and 1927).

Working with sea water and some natural waters, the author found it necessary to measure the carbon dioxide liberated at the 2.0 cc. volume in the reaction chamber instead of the 0.5 cc. volume. Table II gives factors for converting such results to cubic centimeters per liter of carbon dioxide when such a procedure is necessary.

#### DESCRIPTION OF THE ANALYSES IN TABLE III

The water used in this work was made up from distilled water to contain 50 mg. each of  $\text{NaNO}_3$ ,  $\text{MgSO}_4$ , and  $\text{K}_2\text{SO}_4$  and 100 mg. of  $\text{CaCl}_2$  per liter. This water tested free from nitrites. Samples 1-8; inclusive, represent duplicate analyses by the Winkler and modified Van Slyke methods for dissolved oxygen. The Winkler method used was that described in the *Official and Tentative Methods of the Association of Official Agricultural Chemists* (1925).

Samples 9-12, inclusive, represent duplicate analyses by the Van Slyke method for carbon dioxide. Two liters of water were taken as sample A, and a liter of this was siphoned into a volumetric flask containing a weighed amount of sodium carbonate as sample B. By the difference in the  $\text{CO}_2$  content between A and B, the amount of  $\text{CO}_2$  recovered was determined and compared with that added in the form of carbonate.

Samples 13 and 14 represent determinations in which both oxygen and carbon dioxide from the same sample were compared by the methods described above. Winkler determinations were run on both the A and B samples in this case.

#### SUMMARY

1. A modified Van Slyke method for the analysis of dissolved  $\text{CO}_2$  and  $\text{O}_2$  is described, and  $\text{O}_2$  determinations by this method are compared with those by the Winkler method.

2. A 5 cc. sample is required. An analysis can be completed in  $\frac{1}{2}$  hour. The accuracy with  $\text{CO}_2$  is 0.1 cc., and with  $\text{O}_2$  is 0.06 cc., per liter of water.



TABLE III  
SUMMARY OF ANALYSIS\*  
(Values expressed in cc. per liter)

| SAMPLE<br>NO. | WINKLER<br>METHOD<br>O <sub>2</sub> | MODIFIED VAN SLYKE METHOD |                 |       |           |
|---------------|-------------------------------------|---------------------------|-----------------|-------|-----------|
|               |                                     | O <sub>2</sub>            | CO <sub>2</sub> |       |           |
|               |                                     |                           | Analysis        | Added | Recovered |
| 1.....        | 5.93<br>5.99                        | 5.96<br>5.93              | .....           | ..... | .....     |
| 2.....        | 5.90<br>5.85                        | 5.88<br>5.86              | .....           | ..... | .....     |
| 3.....        | 4.83<br>4.80                        | 4.85<br>4.83              | .....           | ..... | .....     |
| 4.....        | 5.60<br>5.65                        | 5.65<br>5.66              | .....           | ..... | .....     |
| 5.....        | 2.22<br>2.28                        | 2.26<br>2.30              | .....           | ..... | .....     |
| 6.....        | 3.45<br>3.53                        | 3.51<br>3.55              | .....           | ..... | .....     |
| 7.....        | 2.46<br>2.49                        | 2.48<br>2.49              | .....           | ..... | .....     |
| 8.....        | 3.72<br>3.76                        | 3.74<br>3.78              | .....           | ..... | .....     |
| 9A.....       |                                     |                           | 2.79<br>2.81    | ..... | .....     |
| 9B.....       |                                     |                           | 25.73<br>25.71  | 22.68 | 22.92     |
| 10A.....      |                                     |                           | 2.09<br>2.03    | ..... | .....     |
| 10B.....      |                                     |                           | 29.60<br>29.51  | 27.99 | 27.50     |
| 11A.....      |                                     |                           | 1.92<br>1.95    | ..... | .....     |
| 11B.....      |                                     |                           | 25.40<br>25.43  | 23.52 | 23.48     |
| 12A.....      |                                     |                           | 2.19<br>2.22    | ..... | .....     |

TABLE III—Continued

| SAMPLE NO.    | WINKLER METHOD O <sub>2</sub> | MODIFIED VAN SLYKE METHOD |                 |       |           |
|---------------|-------------------------------|---------------------------|-----------------|-------|-----------|
|               |                               | O <sub>2</sub>            | CO <sub>2</sub> |       |           |
|               |                               |                           | Analysis        | Added | Recovered |
| 12B . . . . . |                               |                           | 28.34<br>28.37  | 26.15 | 26.31     |
| 13A . . . . . | 4.96<br>4.93                  | 5.15<br>5.13              | 1.89<br>1.85    |       |           |
| 13B . . . . . | 4.80<br>4.81                  | 5.02<br>5.04              | 31.17<br>31.12  | 29.23 | 29.26     |
| 14A . . . . . | 5.03<br>5.01                  | 4.08<br>4.95              | 2.27<br>2.23    |       |           |
| 14B . . . . . | 5.08<br>5.08                  | 5.07<br>5.04              | 29.30<br>29.35  | 27.42 | 27.08     |

Oxygen: Mean diff.=0.038 cc. per liter  
Probability=0.1452

Carbon dioxide: Mean diff.=0.073 cc.  
per liter†  
Probability=0.4558

\* In each case the B sample represents 1 liter of the A sample, to which has been added a weighed portion of Na<sub>2</sub>CO<sub>3</sub>.

† A probability of greater than 0.05 has no statistical significance.

## LITERATURE CITED

- SENDROY, J., JR. 1931. Monometric determination of hemoglobin by the oxygen capacity method. *Jour. Biol. Chem.*, **91**:307-23.
- SKINNER, W. W. 1925. Official and tentative methods. Association of Official Agricultural Chemists, pp. 83-114.
- VAN SLYKE, D. D. 1927. A note on a portable form of the monometric gas apparatus and on certain points in the technique of its use. *Ibid.*, **73**:121-26.
- VAN SLYKE, D. D., and NEILL, J. M. 1924. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. *Jour. Biol. Chem.*, **61**:523-73.
- VAN SLYKE, D. D., and SENDROY, J., JR. 1927. Carbon dioxide factors for the manometric blood gas apparatus. *Ibid.*, pp. 127-44.





FURTHER ANALYSIS OF THE PROTECTIVE VALUE OF  
BIOLOGICALLY CONDITIONED FRESH WATER FOR  
THE MARINE TURBELLARIAN, *PROCERODES*  
*WHEATLANDI*. IV. THE EFFECT OF  
CALCIUM

R. B. OESTING

(From the Marine Biological Laboratory and the University of Chicago)

In a series of studies (1928, 1929, 1933) Allee has shown that the marine turbellarian, *Procerodes wheatlandi*, resists cytolysis longer in extremely hypotonic water, other conditions being equal, if such water has been biologically conditioned than if it lacks biological contamination. These relations hold even though the total concentration of electrolytes, as measured by the conductivity method, is the same. The balance of electrolytes is necessary in such experimental tests, since an increase in their concentration in the proportions found in sea water likewise confers protection.

Protective biological conditioning results when other *Procerodes* have previously been exposed to the fresh water, and especially so if some few *Procerodes* have recently died and disintegrated in it. The protective action of this biologically conditioned fresh water is maintained after dialysis to the same conductance as that of extremely dilute sea water controls. Water extracts of marine amphipods and of fresh water planarians, *Euplanaria novangliae* (= *Planaria maculata*), show these same effects, as does water from cultures of the latter and water from sterilized hay infusions of a practically pure culture of *Paramecium*. The protective effects are not due to pH differences, or to a depressing action such as is produced by exposure to dilute alcohol or to ethyl urethane. The protective agent is not adsorbed by activated charcoal, or by coagulated egg white.

The onset of cytolysis was determined in these experiments by inspection with a hand lens. The results have been confirmed by histological studies on worms taken from Allee's 1933 experiments (Fowler, 1935).

Pantin (1931*a* and *b*) and Weil and Pantin (1931) have studied the adaptation of *Procerodes* (= *Gunda*) *ulvae* to fresh waters. Their results bear on our problem since the two species are closely related taxonomically and in habitat toleration. *P. ulvae* invades small streams

where conditions are suitable; it may extend up these to, or slightly above, mean high water at neap tides (Ritchie, 1934). The reaction of this planarian in fresh waters varies with the chemical composition of the water. The worms swell less and recover more completely on their return to sea water if placed in fresh waters rich in calcium rather than in calcium-deficient waters. In nature, Ritchie (1934) has found them living in fresh water with as little as 5 mgm. per liter of calcium for as long as five days when periods of calm weather, with the resulting lack of salt splash, coincide with neap tides.

#### MATERIALS AND METHODS

*P. wheatlandi* were collected daily from the same locality as in the previous studies of Allee. *Euplanaria novangliae* were collected from a fresh water pond known to be low in salt content; the pond water used came from the same pond which was also the source of these planarians and of pond water in Allee's latest work (1933).

The interval before the beginning of cytolysis in hypotonic waters depends on several factors, among which are the following: Small worms begin and undergo cytolysis more rapidly than do larger ones; worms long in the laboratory are less resistant than are those freshly collected; the more often the medium is renewed, the sooner cytolysis begins. The higher the temperature, or the lower the specific conductance of the water, the less the resistance of *Procerodes* (cf. Allee, 1933).

Assays of the protective value of various media were made, keeping these factors as nearly identical as possible for the worms whose resistance was being comparatively tested except that variations in specific conductance were used as a tool in the analyses. The worms were first isolated in a small drop of sea water on the curved bottom of the salt cellar type of watch glass; they were then washed five times with the respective media into which they were to be placed. The worms were paired for comparative assays before the test began; the members of each pair were selected for similarity in size, activity, and laboratory age. After the washings, 2 cc. of the respective media were run over each worm; this liquid was renewed every one, two, or four hours, depending on the severity of the media and the laboratory age of the worms. The survivals were all tested at room temperature in a room with north exposure which was not subjected to rapid changes in temperature. The highest temperature recorded for the summer in this room was 25.5° C. Examinations were made every fifteen minutes with a ten-power hand lens until the beginning of fatal cytolysis was noted. Ten worms were isolated into each type of media being tested in an assay.

Conductivity measurements were made using a No. 7651 Leeds and Northrup potentiometer and a Washburn type conductivity cell designed for solutions with low conductance. Specific conductances are given in mhos  $\times 10^5$ . Allee has previously given conductivity measurements in terms of ohms resistance. His data may be compared with those given in the present report by the use of the following data: under the conditions given, 6,000 ohms  $= 5.42 \times 10^{-5}$  mhos; 2,500 ohms  $= 13.05 \times 10^{-5}$  mhos and 1,700 ohms  $= 19.20 \times 10^{-5}$  mhos.

Calcium analyses were made using the Van Slyke and Sendroy (1929) method. The accuracy of this method to within the one per cent claimed for it was confirmed by tests on the recovery of calcium added to distilled water and to the other media used in this work.

TABLE I

*The Protective Action of Calcium for Procerodes Isolated into Extremely Hypotonic Water*

| Experiment No. | Medium | Calcium added      |                | Calcium not added |                    | Difference in hours |
|----------------|--------|--------------------|----------------|-------------------|--------------------|---------------------|
|                |        | Mhos $\times 10^5$ | Hours survived | Hours survived    | Mhos $\times 10^5$ |                     |
| 5              | Tap    | 8.38               | 2.74           | 1.80              | 7.30               | 0.94                |
| 6              | Sea    | 30.12              | 2.23           | 1.25              | 28.69              | 0.98                |
| 6a             | Tap    | 8.83               | 3.95           | 2.43              | 7.15               | 1.52                |
| 8              | Tap    | 21.29              | 9.33           | 4.00              | 7.07               | 5.33                |
| 8a             | Sea    | 40.80              | 9.31           | 4.03              | 26.53              | 5.28                |
| 9              | Pond   | 5.02               | 7.20           | 3.30              | 4.79               | 3.90                |
| 10             | Pond   | 5.50               | 5.50           | 3.55              | 4.79               | 1.95                |
| 10a            | Pond   | 6.27               | 15.80          | 5.32              | 4.56               | 10.48               |
| 11             | Pond   | 7.43               | 3.23           | 2.14              | 4.79               | 1.09                |
| 12             | Pond   | 7.13               | 2.63           | 2.20              | 4.79               | 0.43                |
| 13             | Pond   | 5.40               | 4.34           | 3.00              | 4.79               | 1.34                |
| 21             | Pond   | 6.25               | 5.98           | 4.08              | 4.79               | 1.90                |
| Averages       |        |                    | 6.02           | 3.09              |                    | 2.92                |

Statistical probability . . . . . 0.005

#### EXPERIMENTAL RESULTS

Calcium, as  $\text{CaCl}_2$ , was added to tap, pond and to extremely hypotonic sea water in a group of twelve experiments with results which are summarized in Table I. In each assay, the worms survived longer, on the average, in the water to which calcium had been added than did control worms in otherwise similar water. The amount of calcium used differed in different tests; in all cases the increase in specific conductance is a measure of the calcium added. Further comparisons can be made from the fact that in Experiment 8, 1 cc. of M/10  $\text{CaCl}_2$  was

added to 200 cc. of tap water and in Experiment 8a the same amount was added to 200 cc. of 1:300 sea water.

Although varying amounts of calcium were added and further variations were caused by differences in laboratory age and in the number of changes of the media during the assays, when the data given in Table I are considered as twelve paired experiments, the difference in survival of 2.92 hours has a statistical probability of 0.005.<sup>1</sup> The coefficient of correlation between the differences in the amount of calcium as measured by specific conductance and the differences in the resistance of the worms is 0.3691; this is statistically significant.

TABLE II

*Relative survival of Procerodes isolated in tap water and in dilute sea water.* Time is given in hours and each value is the average for 10 worms; specific conductance is in mhos.

| Experiment No. | Dilute sea water |                         | Tap water |                         |
|----------------|------------------|-------------------------|-----------|-------------------------|
|                | Survival         | Sp. cond. $\times 10^5$ | Survival  | Sp. cond. $\times 10^5$ |
| 1              | 4.825            | 19.07                   | 5.725     | 6.64                    |
| 2              | 6.667            | 20.14                   | 8.566     | 6.38                    |
| 3              | 4.697            | 25.90                   | 7.865     | 6.79                    |
| 4              | 2.768            | 28.69                   | 2.100     | 7.82                    |
| 5              | 1.535            | 27.95                   | 1.800     | 7.30                    |
| 6              | 1.250            | 28.69                   | 2.425     | 7.15                    |
| 7              | 7.875            | 39.75                   | 8.150     | 7.07                    |
| 8              | 4.775            | 26.53                   | 4.000     | 7.07                    |
| Averages       | 4.299            | 27.09                   | 5.079     | 7.03                    |

The relatively greater protection from hypotonic water furnished by calcium in comparison with that given by other electrolytes in the proportions found in sea water is illustrated by the data summarized in Experiments 8 and 8a, Table I, and the further data given in Table II. In Experiments 8 and 8a, despite the differences in conductance, there is no significant difference in survival of worms in tap water or in 1:300 sea water and again there is no significant difference in survival in these two waters after the same amount of calcium is added. There is a decidedly significant difference between the survival of the worms in either water before and after the addition of calcium.

The waterworks of Falmouth, which supplies the tap water used in these experiments, now uses a lime treatment which materially increases the calcium content. This treatment was started since Allee's last pre-

<sup>1</sup> Values of 0.05 or less are usually considered statistically significant. "Student's" method of paired comparisons was used in all statistical analyses.



ceding experiments. The presence of this calcium probably accounts for the markedly greater protection of *Procerodes* in this water over that which would be expected from its low specific conductance. The dilutions of sea water contained approximately four times as much electrolytes as did the tap water; rough calculations of the calcium content from analyses furnished by the Falmouth waterworks showed this to be practically the same in the two. The survival of the *Procerodes* isolated in the two types of water was approximately the same in both:

TABLE III

*Survival of Procerodes in planarian-conditioned fresh water as compared with that shown in different control media.* Ten worms were assayed in each experiment except No. 13 in which eight were used.

| Experiment No. | Hours of survival                |                  |                                       |                              |
|----------------|----------------------------------|------------------|---------------------------------------|------------------------------|
|                | I<br>Planarian-conditioned water | II<br>Pond water | III<br>Pond water + CaCl <sub>2</sub> | IV<br>Pond water + sea water |
| 9              | 4.025                            | 3.300            | 7.900                                 | —                            |
| 10             | 3.925                            | 3.550            | 4.650                                 | —                            |
| 11             | 3.650                            | 2.650            | 3.725                                 | 2.150                        |
| 12             | 2.525                            | 2.275            | 2.725                                 | 2.200                        |
| 13             | 4.156                            | 2.594            | 4.219                                 | 3.188                        |
| 20             | 1.525                            | 0.750            | 1.350                                 | 1.225                        |
| 21             | 5.250                            | 2.925            | 5.975                                 | 4.075                        |
| Means          | 3.579                            | 2.578            | 4.363                                 | 2.568                        |

| Statistical analysis |            |  |             |       |
|----------------------|------------|--|-------------|-------|
|                      | Difference |  | Probability | Cases |
| I—II                 | 0.9900     |  | 0.0000      | 68    |
| III— I               | 0.9375     |  | 0.0004      | 68    |
| III— II              | 1.7941     |  | 0.0000      | 68    |
| I—IV                 | 0.8490     |  | 0.0010      | 48    |
| IV— II               | 0.3125     |  | 0.2508      | 48    |
| III—IV               | 1.0313     |  | 0.0000      | 48    |

the mean difference of 0.78 hours longer survival in tap water lacks statistical significance ( $P=0.133$ ).

It is evident from these data that calcium delays the onset of cytolysis when *Procerodes* are isolated in extremely hypotonic fresh waters. Any factor which would tend to increase the amount of calcium present then, should confer such protection. This leads to the fundamental question of the present inquiry: Is the protective value of biologically conditioned water to be attributed to a differential increase in calcium

which accompanies conditioning? It is at once obvious that the addition of sea water to a control sample of fresh water until its total electrolyte content equals that of a biologically conditioned sample as measured by the conductivity method, while furnishing a basis for an adequate check on protection due to increased osmotic pressure, gives inadequate control over any specific electrolyte such as calcium. It is necessary to test directly for the amount of calcium present and to confirm its effectiveness in biologically conditioned water.

Unless otherwise stated, the amount of biological conditioning used was that furnished by 200 *Euplanaria novangliæ* living in one liter of fresh pond water for approximately 44 hours, or its equivalent obtained by using fewer worms for a longer period. Sea water and calcium chloride were added respectively to other portions of fresh pond water until the specific conductance of all three solutions was the same. As an additional control, except in Experiments 9 and 10, a sample of untreated pond water was assayed together with the above media. The average survival times of *Procerodes* from seven such experiments are shown in Table III.

TABLE IV

Calcium analyses of assayed samples of media from Table III; results shown in mgm. calcium per liter.

|                           | I<br>Planarian-conditioned water | II<br>Pond water | III<br>Pond water + CaCl <sub>2</sub> | IV<br>Pond water + sea water |
|---------------------------|----------------------------------|------------------|---------------------------------------|------------------------------|
| Calcium content . . . . . | 2.072                            | 0.809            | 3.410                                 | 1.018                        |

These data, together with the statistical analyses, show that planarian-conditioned water has a protective value lying between that of pond water plus sea water and pond water plus calcium chloride when all are of the same electrolytic content. When, however, the amount of calcium present in the planarian culture water (Table IV) was determined and calcium chloride was added to pond water to bring the calcium content to that of the planarian-conditioned water, no difference could be detected between the effectiveness of these two waters in protecting the *Procerodes* isolated in them. These results are summarized, together with a statistical analysis, in Table V.

To determine whether any factor other than calcium might be involved in the protection furnished by planarian-conditioning, a medium

TABLE V

*Procerodes* survival in pond water and planarian-conditioned pond water with the same calcium content.

| Experiment No. | Hours of survival                |                  |                               |                                      |
|----------------|----------------------------------|------------------|-------------------------------|--------------------------------------|
|                | I<br>Planarian-conditioned water | II<br>Pond water | III<br>Pond water + sea water | IV<br>Pond water + CaCl <sub>2</sub> |
| 26             | 5.250                            | 5.650            | 3.850                         | 8.300                                |
| 27             | 1.875                            | 2.150            | 1.150                         | 3.500                                |
| Means          | 3.563                            | 3.900            | 2.500                         | 5.900                                |

Statistical analysis

|        | Difference | Probability | Cases |
|--------|------------|-------------|-------|
| I- II  | -0.3375    | 1.0000      | 20    |
| I-III  | 1.0625     | 0.0396      | 20    |
| IV- I  | 2.3375     | 0.0052      | 20    |
| II-III | 1.4000     | 0.0230      | 20    |
| IV- II | 2.0000     | 0.0344      | 20    |
| IV-III | 3.4000     | 0.0000      | 20    |

was made up to represent a synthetic river water (Henderson, 1913, p. 113). To each liter of water the following salts were added:

100 mgm. CaCl<sub>2</sub>  
 50 mgm. MgSO<sub>4</sub>  
 25 mgm. NaCl  
 10 mgm. KCl

This water in full, half- and quarter-strengths, when conditioned by planarians, showed no change in the total electrolytic content; likewise there was no protection for *Procerodes* when compared by the usual assay to the survival shown in similarly treated but unconditioned synthetic river water. The results of these experiments together with a statistical analysis are summarized in Table VI. From all this evidence, we can safely conclude that an increase in calcium is the factor furnishing the protection to *Procerodes* in planarian-conditioned pond water.

Allee's previous work had shown that water extracts of *Procerodes* themselves furnished a potent protection for other worms of the same species isolated in extremely hypotonic media. The relation between this protection and the calcium content of the water was also tested.

TABLE VI

A summary of tests which indicate that the protection furnished by calcium is sufficient to account for the protective value of planarian-conditioned water. Each of the survival times given is the average for ten worms.

| Experiment number                   | Hours survival   |         | Amount of conditioning       | Laboratory age of <i>Procerodes</i> | Mhos $\times 10^3$ | Media strength | Media changed | Difference |
|-------------------------------------|------------------|---------|------------------------------|-------------------------------------|--------------------|----------------|---------------|------------|
|                                     | Planaria culture | Control |                              |                                     |                    |                |               |            |
| 14                                  | 10.225           | 5.625   | 100 worms in 250 cc. 16 hrs. | 1 day                               | 48.70              | Full           | Every hour    | +4.60      |
| 15                                  | 6.550            | 6.800   | 100 worms in 500 cc. 44 hrs. | 2 days                              | 48.70              | Full           | Every hour    | -0.25      |
| 16                                  | 21.75            | 19.30   | 14 worms in 250 cc. 68 hrs.  | over 5 days                         | 48.70              | Full           | Every hour*   | +2.45      |
| 17                                  | 5.65             | 6.30    | 200 worms in 500 cc. 20 hrs. | 3 days                              | 26.15              | Half           | Every 2 hours | -0.65      |
| 18                                  | 6.95             | 7.15    | 100 worms in 500 cc. 44 hrs. | 4 days                              | 26.15              | Half           | Every 2 hours | -0.20      |
| 19                                  | 7.00             | 6.20    | 60 worms in 500 cc. 68 hrs.  | 4 days                              | 26.15              | Half           | Every 2 hours | +0.80      |
| 23                                  | 5.50             | 6.90    | 200 worms in 500 cc. 20 hrs. | 1 day                               | 12.32              | Quarter        | Every 2 hours | -1.90      |
| 24                                  | 8.90             | 9.35    | 100 worms in 500 cc. 44 hrs. | 2 days                              | 12.32              | Quarter        | Every 2 hours | -0.45      |
| Average..... +0.55                  |                  |         |                              |                                     |                    |                |               |            |
| Statistical Probability..... 0.4500 |                  |         |                              |                                     |                    |                |               |            |

\* Media exhausted after six changes.



*Procerodes* extract was prepared as in the earlier work. After five washings with distilled water, 500 *Procerodes* were boiled in distilled water for about 15 minutes. The beaker was then covered and set aside for 24 hours. Controls were made up to the same specific conductance as the extract by adding calcium chloride to distilled water and sea water to distilled water respectively. All three media were then assayed for their protective value to *Procerodes* and were then analyzed for calcium. It is evident from an examination of Tables VII and VIII which summarize these data that calcium is also the factor in *Procerodes* extracts

TABLE VII

The protective value of *Procerodes* extracts for *Procerodes* isolated in hypotonic media; each survival time given is the average for ten worms.

| Experiment No. | Hours survival                 |                                                  |                                           |
|----------------|--------------------------------|--------------------------------------------------|-------------------------------------------|
|                | I<br><i>Procerodes</i> extract | II<br>Dist. H <sub>2</sub> O + CaCl <sub>2</sub> | III<br>Dist. H <sub>2</sub> O + sea water |
| 28             | 6.150                          | 6.350                                            | 4.150                                     |
| 29             | 2.100                          | 3.000                                            | 1.750                                     |
| Means          | 4.125                          | 4.675                                            | 2.950                                     |
|                | Statistical analysis           |                                                  |                                           |
|                | Difference                     | Probability                                      | Cases                                     |
|                | I- II                          | 0.550                                            | 0.5280                                    |
|                | I-III                          | 1.175                                            | 0.0344                                    |
| II-III         | 1.725                          | 0.0050                                           |                                           |

TABLE VIII

Calcium analyses of assayed samples from Table VII; results shown in mgm. per liter.

|                           | <i>Procerodes</i><br>extract | Dist. H <sub>2</sub> O<br>+ CaCl <sub>2</sub> | Dist. H <sub>2</sub> O<br>+ sea water |
|---------------------------|------------------------------|-----------------------------------------------|---------------------------------------|
| Calcium content . . . . . | 7.20                         | 8.66                                          | 0.42                                  |

which furnishes the observed protection for *Procerodes*. Since making a water extract of *Procerodes* is a short-cut method for preparing *Procerodes*-conditioned water, it is unnecessary to examine this possibility for further confirmatory evidence that an increase in calcium is the fac-

tor furnishing protection to *Procerodes* in biologically conditioned fresh waters.

### DISCUSSION

These experiments were carried on by essentially the same methods used by Allee in his preceding studies and support his results wherever similar points were being tested. Specifically, in addition to more general relations, these experiments confirm the earlier findings, that, other conditions being equal, *Procerodes* survive longer (1) if the osmotic pressure is increased by the addition of sea water; (2) if the hypotonic water is biologically conditioned (*a*) by the presence of living fresh water planarians or (*b*) by the presence of water extracts of freshly killed *Procerodes*.

In addition, the earlier results are extended by the demonstration that there is more calcium added than would be expected from its proportionate concentration in sea water and that calcium has protective value greater than would be expected from its osmotic effect. There is nothing in the earlier experiments which is out of harmony with the present findings.

In certain of Allee's work (1929, 1933) the conditioned water was dialyzed to bring the specific conductance to the desired experimental level. Such dialyzed solutions were definitely protective. This protection can be explained adequately in the light of the present work if one considers the amounts of calcium introduced into conditioned water, for example, by *Procerodes* extracts (cf. Table VIII). Even if one assumes that the collodion membranes used were freely permeable to calcium, the calcium content could be reduced by more extreme dialysis than that used and still leave enough calcium to be definitely protective as compared with a similarly hypotonic sea water control.

The demonstration that the protective action of these biologically conditioned solutions is due to the increased calcium content in so far as the resistance of *Procerodes wheatlandi* to hypotonic water is concerned, brings this phenomenon into line with the greater resistance shown by *Procerodes* (= *Gunda*) *ulvæ* to fresh water when the calcium content is high. This is hardly the place, nor is *Procerodes* necessarily the most favorable material for an inquiry into the mechanism of calcium protection under these conditions.

The analyses of Pantin and his associates and of Beadle (1931, 1934) indicate that *P. ulvæ* lives in osmotic equilibrium with sea water and reaches a steady physiological state in certain fresh waters. This steady state is not the result of simple osmotic balance but is dependent

largely on the calcium content of the water. It is maintained in part by a change in the permeability of the surface epithelium and is accompanied by increased respiration; it cannot be maintained indefinitely in extremely hypotonic waters. It seems highly probable (cf. Weil and Pantin, 1931) that the mechanism with these planarians is related to that in other cells in certain of which it has been shown, *Arbacia* eggs for example (McCutcheon and Lucke, 1928), that calcium decreases cell permeability to water and (Heilbrunn, 1930) favors membrane formation at a broken surface. The protection in these cases appears to be due to a decreased water intake rather than to a decreased leaching out of materials essential for continued existence.

We have not investigated the range of concentrations of calcium which would delay cytolysis in *Procerodes* placed in hypotonic water; in fact, we have established neither the upper nor the lower effective concentration. We know that an increase in specific conductance produced by the addition of calcium chloride equal to  $1.08 \times 10^{-5}$  mhos will give measurable protection. A rough calculation shows that this is approximately equivalent to the introduction of M/2600  $\text{CaCl}_2$ . This approaches the lower limit of effectiveness under the conditions of our experiments. Buchanan (1935) reports that complete cytolysis readily occurs in *Euplanaria dorotocephala* in distilled water while in distilled water solutions of  $\text{CaCl}_2$  there is no cytolysis in concentrations from M/500 to M/40,000 and cytolysis is distinctly delayed in a dilution of M/100,000. He found a detectable protective action in a M/1,000,000 solution.

We have been engaged in investigating the relation between calcium and the protective action of biological conditioning in extremely hypotonic waters when the calcium content is relatively low. We have little evidence regarding the effectiveness or non-effectiveness of such conditioning when the calcium is high. There are some indications in the first three significant lines in Table VI where full strength synthetic river water was used, that such conditioning may be effective. If so, some other mechanism than an increase in calcium is probably acting. These conditions approximate those found when a hard water stream from a limestone region flows into the ocean (cf. Breder, 1934). There is evolutionary and ecological as well as physiological interest in the relations between numbers present and the effectiveness of the invasion of such waters. Our present work does not deal with this question. However, it does show that marine invasions by animals with the physiological requirements of *Procerodes* into such relatively soft fresh waters as we have investigated is definitely favored by the presence in these waters of calcium excretors such as *Euplanaria novangliae* has been shown to be.

We have no information concerning the actual importance of such conditioning under natural conditions.

Throughout these studies there has been evidence of two opposing effects of numbers upon the resistance of these worms to fresh water; (a) the protective effect of freshly conditioned water or of numbers of animals present and (b) the harmful effect of numbers which shows up in conditioned media that have been allowed to go stale. At the osmotic level of these experiments, the protective effect is due to an increase in calcium and while the harmful effect has not been analyzed, it is a good *a priori* guess that the observed ill effects are associated with the accumulation of waste products of metabolism or to decomposition products of these.

The series of experiments of which this is the fourth report, were begun and have been prosecuted primarily in an investigation of a phase of mass physiology associated with animal aggregations (cf. Allee, 1931, 1934). The original impetus towards these particular experiments came from the observations of Drzewina and Bohn (1920, 1928) that the marine turbellarian *Convoluta roscoffensis* survives longer in hypotonic water if present in numbers than if isolated. This protection they attributed to the more rapid production of some sort of auto-protective substance by the group than would be possible for a single individual. This suggestion is now seen to have been essentially correct and, under the conditions of our experiments, to be composed of nothing more mysterious than calcium.

#### SUMMARY

1. An increase in the calcium content of extremely hypotonic water, when the calcium content is less than that found in sea water, delays the onset of fatal cytolysis for *Procerodes wheatlandi* isolated in such media.

2. Other electrolytes, even when increased appreciably, do not show this same protection; however, some protection of an osmotic nature is apparent.

3. The increase of calcium in biologically conditioned fresh waters is adequate to explain their observed protection for *Procerodes*.

#### LITERATURE CITATIONS

- ALLEE, W. C., 1928. Studies in Animal Aggregations: Mass protection from fresh water for *Procerodes*, a marine turbellarian. *Jour. Exper. Zool.*, **50**: 295.
- ALLEE, W. C., 1929. Studies in Animal Aggregations: Mass protection from hypotonic sea-water for *Procerodes*, a marine turbellarian, with total electrolytes controlled. *Jour. Exper. Zool.*, **54**: 349.
- ALLEE, W. C., 1931. Animal Aggregations: A Study in General Sociology. University of Chicago Press.

- ALLEE, W. C., 1933. Studies in Animal Aggregations: Further analysis of the protective value of biologically conditioned fresh water for the marine turbellarian, *Procerodes*. *Physiol. Zool.*, **6**: 1.
- ALLEE, W. C., 1934. Recent Studies in Mass Physiology. *Biol. Rev.*, **9**: 1.
- BEADLE, L. C., 1931. The Effect of Salinity Changes on the Water Content and Respiration of Marine Invertebrates. *Jour. Exper. Biol.*, **8**: 211.
- BEADLE, L. C., 1934. Osmotic Regulation in *Gunda ulvæ*. *Jour. Exper. Biol.*, **11**: 382.
- BOHN, G., AND A. DRZEWINA, 1920. Variations de la sensibilité à l'eau douce des *Convoluta*, suivant les états physiologiques et le nombre des animaux en expérience. *Compt. Rend. Acad. Sci.*, **171**: 1023.
- BREder, C. M., JR., 1934. Ecology of an Oceanic Fresh-Water Lake, Andros Island, Bahamas, with Special Reference to its Fishes. *Zoologica*, **18**: 57.
- BUCHANAN, J. W., 1935. An Analysis of Physiological States Responsible for Antero-posterior Disintegration in *Planaria dorotocephala*. *Biol. Gen.*, in press.
- DRZEWINA, A., AND G. BOHN, 1928. Les *Convoluta*. *Ann. Sci. Nat. Zool.*, **X**, Ser. **11**: 299.
- FISHER, R. A., 1925. Expansion of "Students'" Integral in Powers of  $N^{-1}$ . *Metron*, **5**: 109.
- FOWLER, LILLIAN HENDERSON, 1935. Relative Effect of Biologically Conditioned and Other Extremely Hypotonic Water on the Tissues of the Marine Turbellarian, *Procerodes wheatlandi*. *Physiol. Zool.*, **8**: No. 2.
- HEILBRUNN, L. V., 1930. The Action of Various Salts on the First Stage of the Surface Precipitation Reaction in *Arbacia* Egg Protoplasm. *Protoplasma*, **11**: 558.
- HENDERSON, L. J., 1913. *The Fitness of the Environment*. Macmillan.
- MCCUTCHEON, M., AND B. LUCKÉ, 1928. The Effect of Certain Electrolytes and Non-electrolytes on Permeability of Living Cells to Water. *Jour. Gen. Physiol.*, **12**: 129.
- PANTIN, C. F. A., 1931a. The Adaptation of *Gunda ulvæ* to Salinity: I. The environment. *Jour. Exper. Biol.*, **8**: 63.
- PANTIN, C. F. A., 1931b. The Adaptation of *Gunda ulva* to Salinity: III. The electrolytic exchange. *Jour. Exper. Biol.*, **8**: 82.
- RITCHIE, A. D., 1934. Habitat of *Procerodes ulvæ*. *Jour. Mar. Biol. Ass.*, **19**: 663.
- "STUDENT," 1925. New Tables for Testing the Significance of Observations. *Metron*, **5**: 105.
- VAN SLYKE, D. D., AND J. SENDROY, JR., 1929. Gasometric Determination of Oxalic Acid and Calcium, and its Application to Serum Analysis. *Jour. Biol. Chem.*, **84**: 217.
- WEIL, E., AND C. F. A. PANTIN, 1931. The Adaptation of *Gunda ulvæ* to salinity. II. The water exchange. *Jour. Exper. Biol.*, **8**: 73.









# NOTE ON A MODIFIED METHOD DETERMINING THE NITROGEN DISTRIBUTION OF PROTEINS\*

R. B. OESTING

Department of Physiological Chemistry and Pharmacology  
The University of Chicago

This method makes use of the gasometric micro-Kjeldahl determination of nitrogen described by Van Slyke (1927) combined with the Koch-McMeekin (1924) peroxide digestion of micro-samples. Some slight modifications were necessary to combine these two methods and these changes will be described later. Alpha amino nitrogen was determined in the Koch (1929) modification of the Van Slyke apparatus. The details of the nitrogen distribution method have been modified: ammonia and melanoidin nitrogen were determined by the difference in the total nitrogen content of the sample before and after their removal; the technique of separating the hexone bases has been modified; total nitrogen and alpha amino nitrogen were determined on the same aliquot. This modified method in skilled hands makes possible an accurate study of the protein in less than 50 hours of laboratory work over a period of 5 days.

## MICRO-KJELDAHL METHOD

A sample containing 1 to 5 mg. of nitrogen is digested by the peroxide method using 1.5 cc. of 1:1 sulphuric acid and 30 per cent hydrogen peroxide. It is then transferred quantitatively to a 25 cc. volumetric flask and diluted to the mark. A blank on the reagents is run along with the sample. Five cc. of this sample or blank is then analyzed by the Van Slyke method using 2 cc. of the hypobromite reagent. Correction (*c*) is made from the blank analysis for nitrogen introduced with the various reagents, etc. In my experience the use of  $H_2O_2$  for oxidizing the organic matter does not lead to inconstant results as is claimed by Van Slyke (1927, p. 237).

\* Privately printed.

## ANALYSIS OF PROTEIN

1. (a) In a 500 cc. tared flask hydrolyze about 2 gm. of protein until the  $\alpha$ -amino nitrogen shows no further increase. (b) Run micro-Kjeldahls for total nitrogen on aliquots from the  $\alpha$ -amino nitrogen samples used in 1 (a).

2. Remove the excess HCl by vacuum technique and add just enough  $\text{Ca(OH)}_2$  to make the hydrolysate alkaline to litmus paper. Avoid an excess. Remove the  $\text{NH}_3$  (amide nitrogen) by vacuum distillation for 45 minutes at 40 to 50° C. and again sample the material and determine the total nitrogen by micro-Kjeldahl. The difference in nitrogen content represents amide nitrogen.

3. Filter through a 12 or 15 cm. hardened filter paper into a tared 500 cc. flask and wash free of chlorides. After acidifying with HCl concentrate the filtrate and washings to about 50 cc. by vacuum distillation. Sample by weighing and determine the total nitrogen micro-Kjeldahl. The difference represents melanoidin nitrogen.

4. Transfer by weighing about 25 cc. of the sample to a 100 cc. centrifuge tube and add 4.5 cc. of concentrated HCl and 12.5 cc. of 30 per cent phosphotungstic acid. Then add water to make the volume to 50 to 60 cc. and heat in a large water bath until the precipitate is practically all dissolved. Cool slowly to 20° C. and allow to stand for 48 hours. Wash by decantation with centrifugation using 6 portions of 2.5 per cent phosphotungstic acid in 3.5 per cent HCl cooled to 0° C. Combine the washings and set aside for step 6 below.

5. Wash the precipitate from the centrifuge tube into a tared flask with 1 per cent NaOH and dissolve by adding a few drops at a time of 40 per cent NaOH, the final solution being just alkaline to phenolphthalein. Then add dilute  $\text{H}_2\text{SO}_4$  to just neutralize the excess alkali. Weigh aliquots for amino and total nitrogen determinations.

6. Concentrate the washings from step 4 by vacuum distillation to about 40 cc. in a tared flask and take aliquots by weighing for total nitrogen and  $\alpha$ -amino nitrogen.

The author found that additions of caprylic alcohol in step 2 were sufficiently effective in preventing foaming during the removal of the ammonia. Additions of small amounts of ethyl alcohol were also made at this point. Step 6 of the procedure can be omitted, since



the nitrogen in this fraction can be determined by difference. For further information see Plimmer and Rosedale (1925).

Two separate analyses of Hammarsten's casein gave the following results with this method:

|                                | Per Cent | Per Cent |
|--------------------------------|----------|----------|
| Total nitrogen . . . . .       | 14.89    | 15.04    |
| Alpha amino nitrogen . . . . . | 11.10    | 11.28    |
| Ammonia nitrogen . . . . .     | 1.08     | 1.12     |
| Melanoidin nitrogen . . . . .  | 0.46     | 0.54     |
| Hexone base nitrogen . . . . . | 3.16     | 3.28     |
| Mono amino nitrogen . . . . .  | 10.19    | 10.10    |

These results compare favorably with those reported in the literature and there is no reason to expect otherwise. All the methods employed are of proved value in the laboratories of other workers.

LITERATURE CITED

KOCH, F. C., and McMEEKIN, T. L. 1924. Jour. Amer. Chem. Soc., 46:2066-69.  
KOCH, F. C. 1929. Jour. Biol. Chem., 84:601-3.  
PLIMMER, R. H. A., and ROSEDALE, J. L. 1925. Biochem. Jour., 19:1004-14.  
VAN SLYKE, D. D. 1927. Jour. Biol. Chem., 61:235-48.











